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(54) **FORMULATIONS FOR FACTOR IX**

FORMULIERUNGEN FUER FAKTOR IX

FORMULATIONS S'APPLIQUANT AU FACTEUR IX

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Description

FIELD OF INVENTION

[0001] The present invention relates generally to novel formulations comprising factor IX.

BACKGROUND OF THE INVENTION

[0002] A variety of factors involved in the blood clotting process have been identified, including factor IX, a plasma glycoprotein. A deficiency of factor IX characterizes a type of hemophilia (type B). Treatment of this disease has traditionally involved *intra venous* infusion of human plasma-derived protein concentrates of factor IX. Infusion of blood concentrates involves the risk of transmission of various infectious agents, such as viral hepatitis and HIV, or thromboembolic factors. An alternative method of producing factor IX, by recombinant DNA techniques, has been described in USPN 4,770,999, Kaufman *et al.*, September 13, 1988. The cDNA coding for human factor IX has been isolated, characterized, and cloned into expression vectors. *See,* for example, Choo *et al.*, Nature *299*:178-180 (1982); Fair *et al.*, Blood *64*:194-204 (1984); and Kurachi *et al.*, Proc. Natl. Acad. Sci., U.S.A. *79*:6461-6464 (1982). Thus, through advances in recombinant DNA technology, it has been possible to produce factor IX protein.

[0003] It is desirable to have concentrated forms of bulk protein, *e.g.*, factor IX, which, in turn, may be stored and which are suitable for further manufacture of finished dosage forms of protein. Typically, a purification process for a protein results in concentrating the protein. This concentrated protein, also known as bulk protein, may be in a formulation buffer. Bulk protein, typically at a concentration of about 2 to at least 20 mg/ml, can then be shipped frozen to a fill/finish facility where it is diluted to an appropriate dosage concentration and placed into dosage vials. These diluted samples can be lyophilized, *i.e.*, freeze-dried. The lyophilized samples may be kept in long-term storage and reconstituted at a later time by adding a suitable administration diluent just prior to patient use.

[0004] Protein stability can be affected *inter alia* by such factors as ionic strength, pH, temperature, repeated cycles of freeze/thaw and exposures to shear forces. Active protein may be lost as a result of physical instabilities, including denaturation and aggregation (both soluble and insoluble aggregate formation), as well as chemical instabilities, including, for example, hydrolysis, deamidation and oxidation, to name just a few. For a general review of stability of protein pharmaceuticals, see, for example, Manning, *et al.*, Pharmaceutical Research *6*:903-918 (1989).

[0005] While the possible occurrence of protein instabilities is widely appreciated, it is impossible to predict particular instability problems of a particular protein, Any of these instabilities can result in the formation of a protein, protein byproduct, or derivative having lowered activity, increased toxicity, and/or increased immunogenicity. Indeed, protein precipitation may lead to thrombosis, non-homogeneity of dosage form and amount, as well as clogged syringes. Also, specific to factor IX, there are several post-translational modifications (for example, the gamma carboxylation of certain glutamic acid residues in the N-terminus and the addition of carbohydrate) which may be important in maintaining biological activity and which may be susceptible to modification upon storage. Thus, the safety and efficacy of any pharmaceutical formulation of a protein is directly related to its stability.

[0006] In addition to stability considerations, one generally selects excipients which are or will meet with the approval of various world-wide medical regulatory agencies. The solution should be isotonic and the pH in a physiologically suitable range. The choice and amount of buffer used is important to achieve the desired pH range. Moreover, in the case of factor IX, agents such as "heparin" are to be avoided because of potential interference with clotting time assay analysis and with accurate assessment of thrombogenic potential.

[0007] Currently, there are only two commercially available, carrier-protein-free, plasma-derived factor IX formulations. Alpha Therapeutic Corporation provides lyophilized AlphaNine® SD: comprising heparin, dextrose, polysorbate 80, and tri(n-butyl) phosphate. This preparation is meant to be stored at temperatures between 2° and 8°C. As noted *supra*, heparin is to be avoided as it is an anti-coagulant and tri(n-butyl) phosphate is irritating to mucous membranes; thus, this formulation is less than ideal. Armour Pharmaceutical Company's lyophilized Mononine®: comprising histidine, sodium chloride and mannitol is similarly meant to be stored at 2° to 8°C. The package insert recommends not storing this formulation for greater than one month at room temperature.

[0008] Ideally, formulations developed should also be stable for factor IX bulk storage in high concentration (≥20 mg/ml, for example) which allows for relatively small volumes for fill/finish at the appropriate dose and also allows for alternate methods of administration which may require high protein concentration, *e.g.*, sub *cutaneous* administration. Accordingly, there continues to exist a need in the art for methods for improving factor IX protein stability (and maintaining activity levels) during the concentration process, and the lyophilization process, as well as providing stable formulations during prolonged storage.

BRIEF SUMMARY OF THE INVENTION

[0009] One aspect of the present invention provides novel compositions and methods for providing concentrated preparations of factor IX, useful as bulk drug product. These compositions, either frozen, liquid, or lyophilized, comprise factor IX, a bulking agent, such as glycine, and a cryoprotectant. A preferred factor IX concentration ranges from about 0.1 to at least 20 mg/ml (equivalent to about 20 to at least 4000 U/ml). Preferred bulking agents include glycine, and/or a magnesium, calcium, or chloride salt, preferably ranging in concentration from about 0.5 to 300 mM. A suitable cryoprotectant is sucrose, preferably range in concentration from about 0.5 to 2%. Optionally, these bulk drug product compositions may also contain a surfactant or detergent, such as polysorbate (e.g., Tween-80) ® or polyethyleneglycol (PEG), which may also serve as a cryoprotectant during the freezing step. The surfactant preferably ranges from about 0.005 to 0.05%. Preferably, the concentrations of the excipients provide a combined osmolality of about 250 to 350 milliosmolal (mOsM), preferably about 300 mOsM ± 50 mOsM, and further, may contain an appropriate buffering agent to maintain a physiologically suitable pH *e.g.*, in the range preferably of about 6.0 to 8.0. Buffering agents preferably include histidine, and sodium or potassium phosphate, with a target pH of about 6.5 to 7.5, all at about 5-50 mM.

[0010] Another aspect of the present invention provides formulations of factor IX suitable for administration in a final dosage form, for example, via *intra venous* or *sub cutaneous* injection. Preferred formulations include factor IX concentrations ranging from about 0.1 to at least 20 mg/ml, about 0.5 to 2% sucrose, about 0.1 to 0.3 M glycine, and about 0.005% to 0.02% polysorbate, with histidine as a buffering agent, ranging from about 5 to 50 mM. A preferred lyophilized formulation comprises about 0.1 to at least 10 mg/ml factor IX, about 260 mM glycine, about 1% sucrose, about 0.005% polysorbate, and about 10 mM histidine, at pH 7.0.

DETAILED DESCRIPTION OF THE INVENTION

[0011] As used herein, the terms lyophilization, lyophilized, and freeze-dried include but are not limited to processes including "freezing" a solution followed by "drying", optionally *in vacuo*. As used herein, the term "bulking agent" comprises agents which provide good lyophilized cake properties, which help the protein overcome various stresses (shear/freezing for example) associated with the lyophilization process, and which help to maintain protein activity levels. Exemplary bulking agents include, but are not limited to, glycine, $MgCl_2$, $CaCl_2$, NaCl, and the like. These agents contribute to the tonicity of the formulations. Cryoprotectants also contribute to the tonicity. The term "cryoprotectants" generally includes agents which provide stability to the protein from freezing-induced stresses; however, the term also includes agents that provide stability, *e.g.*, to bulk drug formulations during storage from non-freezing-induced stresses. The cryoprotectant is sucrose. The term "lyoprotectant" includes agents that provide stability to the protein during water removal from the system during the drying process, presumably by maintaining the proper conformation of the protein through hydrogen bonding. The cryoprotectant can also have lyoprotectant effects. While preferred concentrations of cryoprotectant range from about 0.5 to 2%, relatively high concentrations, for example 5%, are suitable with the levels used limited only by those customarily used in clinical practice.

[0012] "Surfactants" generally include those agents which protect the protein from air/solution interface induced stresses and solution/surface induced stresses (*e.g.*, resulting in protein aggregation), and may include detergents such as polysorbate-80 (Tween), for example, 0.005-0.05% (weight/volume), or polyethyleneglycol (PEG), such as PEG8000, for example. Optionally, relatively high concentrations, *e.g.*, up to 0.5%, are suitable for maintaining protein stability; however, the levels used in actual practice are customarily limited by clinical practice.

[0013] The term "buffering agent" encompasses those agents which maintain the solution pH in an acceptable range prior to lyophilization and may include histidine, phosphate (sodium or potassium), tris (tris (hydroxymethyl) aminomethane), diethanolamine, and the like. The upper concentration limits are generally higher for "bulk" protein than for "dosage" protein forms as is readily appreciated by one skilled in the art. For example, while buffer concentrations can range from several millimolar up to the upper limit of their solubility, *e.g.*, histidine could be as high as 200 mM, one skilled in the art would also take into consideration achieving/maintaining an appropriate physiologically suitable concentration. Percentages are weight/weight when referring to solids and weight/volume when referring to liquids. The term "isotonic," 300 ± 50 mOsM, is meant to be a measure of osmolality of the protein solution prior to lyophilization; reconstitution is typically with water for injection (WFI). Maintaining physiological osmolality is important for the dosage formulations. However, for bulk formulations, much higher concentrations can be effectively utilized as long as the solution is made isotonic prior to use. The term "excipients" includes pharmaceutically acceptable reagents to provide good lyophilized cake properties (bulking agents) as well as provide lyoprotection and cryoprotection of the protein, maintenance of pH, and proper conformation of the protein during storage so that substantial retention of biological activity (protein stability) is maintained.

[0014] As used herein, factor IX concentration is conveniently expressed as mg/ml or as U/ml, with 1 mg approximately equal to 200 U/ml ± 100 U/ml.

[0015] The following examples illustrate practice of the invention. These examples are for illustrative purposes only

and are not intended in any way to limit the scope of the invention claimed. The cryoprotectant of the invention is sucrose. Example 1 describes recombinant factor IX in various formulations (all isotonic), followed by lyophilization and storage at three different temperatures for one month. The compositions are reconstituted with water and evaluated for particulate formation, recovery of protein, specific activity, and percent aggregate formation. Example 2 provides further formulations and, Example 3 relates to bulk storage stability of factor IX at a relatively high protein concentration.

Example 1

[0016] Samples are prepared in the formulations set forth in Table I below, at a recombinant factor IX protein concentration of ~0.5 mg/ml (100 U/ml) and an osmolality of 300 ± 50 mOsM. All samples contain a recombinant form of factor IX as purified by conformation specific monoclonal antibody column. The preparation of recombinant factor IX has been described in USPN 4,770,999, Kaufman, *et al.* One suitable purification method is that described in Hrinda, *et al.*, Preclinical Studies of a Monoclonal Antibody - Purified Factor IX, Mononine™ Seminars in Hematology, *28(3)*: 6 (July 1991). Other methods of preparation include the use of conformation-specific monoclonal antibodies as described by Tharakan, *et al.*, "Physical and biochemical properties of five commercial resins for immunoaffinity purification of factor IX." Journal of Chromatography *595*:103-111 (1992); and by Liebman, *et al.*, "Immunoaffinity purification of factor IX (Christmas factor) by using conformation-specific antibodies directed against the factor IX-metal complex." Proc. Nat'l. Acad. Sci., USA *82*:3879-3883 (1985); as well as conventional chromatographic procedures, for example, as described by Hashimoto, *et al.*, "A Method for Systematic Purification from Bovine Plasma of Six Vitamin K-Dependent Coagulation Factors: Prothrombin, Factor X, Factor IX, Protein C, and Protein Z." J. Biochem. *97*:1347-1355 (1985), and Bajaj, P. *et al.* Prep. Biochem. *11*:397 (1981).

Table I

Sample Number	pH	Buffer (10 mM)	Salt (Bulking agent)	Cryo-Lyo protectant
1	7.0	histidine	0.066 M NaCl	3% mannitol
2	7.0	histidine	0.13 M glycine	3% mannitol
3	7.0	potassium phosphate	0.12 M glycine	3% mannitol
4	7.0	potassium phosphate	0.25 M glycine	1 % sucrose
5	7.0	histidine	0.26 M glycine	1% sucrose
6	7.0	histidine	0.25 M glycine, 5 mM Ca^{++}	1% sucrose
7	7.0	sodium phosphate	0.25 M glycine	1% sucrose
8	7.5	potassium phosphate	0.25 M glycine	1% sucrose
9	7.5	sodium phosphate	0.25 M glycine	1% sucrose
10	7.5	tris	0.26 M glycine	1% sucrose
11	7.5	tris	0.25 M glycine, 5 mM Ca^{++}	1% sucrose
12	7.5	tris	0.13 M glycine	3% mannitol
13	7.5	diethanolamine	0.26 M glycine	1% sucrose
14	7.5	diethanolamine	0.13 M glycine	3% mannitol
15	7.5	diethanolamine	0.25 M glycine, 5 mM Ca^{++}	1% sucrose

[0017] Another set of 15 samples is prepared, as above however, containing, in addition, a surfactant, 0.005% Tween-80®. The formulation of sample 1 is that formulation used for commercially available plasma-derived factor IX (Mononine™).

A. Effects of Freeze/Thaw Cycle

[0018] Prior to lyophilization, samples of each formulation are subjected to five freeze-thaw cycles to determine susceptibility to freezing-induced denaturation. A series of -80°C/37°C freeze-thaw cycles (five, for example) prior to lyophilization is a useful "indication" of a protein's susceptibility to increased aggregate formation as may be observed in a lyophilization process and/or during long-term storage. Samples are assayed for the amount of "high molecular weight species" (HMW) present; HMW includes covalent and non-covalent aggregates as measured by SEC-HPLC

and SDS-PAGE (reduced and non-reduced). Samples with Tween-80 ® (0.005%) added have minimal aggregation generated (less than 0.1% HMW increase). Without the addition of surfactant, formulations 1, 6, 11 and 15 show greater than 6% HMW generated and the other formulations had <4% HMW increase.

B. Temperature and Surfactant Effects Over Time

[0019] Prior to lyophilization, each sample (with and without surfactant (Tween (0.005%))) is sterile filtered through a 0.2 μm filter. Half ml volumes are filled into 2 ml lyophilization vials and loaded into a lyophilizer. The vials are frozen for 5.5 hours at -50°C. The shelf temperature is raised to -30°C to begin primary drying and held for 42 hours. The shelf temperature is raised to +25°C over a 1 hour time period, and secondary drying started and held for 15 hours. Vials are stoppered at the conclusion of secondary drying. All formulations exhibit good cake properties, and are all easily reconstituted in ≤30 seconds after water is added. Immediately after lyophilization, samples are evaluated for HMW increase. Most non-Tween containing had ~/- 2% increase. Subsequently, samples are stored at three different temperatures (-80°C, 4°C, and 30°C) for a one month period of time. The percentage HMW increase is expressed as a percent of area (absorbance at 280 nm) from SEC-HPLC after lyophilization. Table II. After one month storage, many non-surfactant-containing formulations give a higher percentage increase HMW ranging from 0 to 25%, which is most apparent at the 30°C storage temperature. In particular, samples 1-3, 12, and 14 give the highest percentage increases.

[0020] While formulations having surfactant added, generally have a lower percentage increase in HMW, *i.e.*, minimization of the freezing-induced aggregation from the lyophilization process itself, long-term lyoprotection further depends on the presence of other excipients. For example, those formulations with sucrose rather than mannitol have a lower percentage increase in HMW. Thus, mannitol formulations 1, 2, 3, 12 and 14, with or without surfactant, give up to a 36% increase in percent HMW.

Table II

SEC-HPLC Change in Percent HMW One Month At Three Temperatures Without Tween® (-T) and With Tween® (+T)

Temp.			-80°C		4°C		30°C	
Sample No.	Time Zero[1]		One Month[2]		One Month[2]		One Month[2]	
	-T	+T	-T	+T	-T	+T	-T	+T
1	1.1	0.0	8.4	-1.0	10.0	-1.0	14.0	18.0
2	1.9	0.1	2.0	0.8	2.0	0.4	8.0	7.5
3	1.4	0.0	2.2	0.1	3.0	-1.5	8.0	3.5
4	0.5	0.1	0.6	-1.5	1.0	-1.9	1.5	-1.0
5	0.9	0.0	3.0	-0.6	4.1	-0.5	4.0	-1.0
6	0.7	0.2	4.4	0.2	4.0	0.1	5.0	0.0
7	0.7	0.2	2.2	-0.1	3.1	-0.1	3.0	-0.2
8	1.6	0.2	0.4	-0.2	2.6	-0.1	0.8	-0.1
9	1.2	0.3	2.0	-0.2	3.5	-0.8	2.0	-0.1
10	1.1	0.1	1.0	-0.4	1.6	-0.8	2.1	-0.2
11	0.3	0.2	0.4	0.0	0.8	-0.1	0.8	0.0
12	0.6	0.1	0.6	-1.5	3.4	-1.0	8.0	6.0
13	0.0	0.0	0.1	0.0	0.8	-0.5	0.8	0.1
14	1.5	0.0	3.0	0.1	5.4	13.0	25.0	36.0
15	0.0	0.0	-1.0	1.0	-1.2	2.0	1.0	1.0

[1] = Time Zero (%) percent change in HMW from "before lyophilization" to "after lyophilization"

[2] = Increase in area %HMW relative to Time Zero value

[0021] The clotting activity and specific activity values for the one month, -80°C, 4°C and 30°C samples are determined. Factor IX activity is determined according to the method of Pittman, D., *et al.*, Blood *79*:389-397 (1992) utilizing

factor IX-deficient blood.

[0022] Little differences in recovery of activity or specific activity are observed at - 80°C or 4°C after one month (with or without surfactant added); however, at 30°C, recovery of activity and specific activity correlates generally with the aggregation results; in other words, a loss of activity is generally observed with increased aggregation, most notably in formulations 1, 2, 3, 12, and 14, where addition of surfactant did not prevent aggregation from occurring over time.

Example 2

[0023] Additionally, two formulations comprising histidine, glycine (with and without surfactant), and 2% sucrose, isotonic, are evaluated and are found to maintain factor IX activity.

[0024] Another set of 10 formulations is prepared as listed in Table III (with an osmolality of 300 ± 50 mOsM), lyophilized as previously described, and placed at -80°C, 4°C, and 30°C for storage and stability analysis at one, three, and four months. All samples have surfactant added, *i.e.*, 0.005% Tween-80.

Table III

Sample Number	pH	Buffer (10 mM)	Glycine	Sucrose %
1	7.0	histidine	0.26 M	1
2	7.0	histidine	0.29 M	0
3	7.0	sodium phosphate	0.25 M	1
4	7.0	potassium phosphate	0.25 M	1
5	7.5	tris	0.26 M	1
6	7.5	potassium phosphate	0.25 M	1
7	7.5	sodium phosphate	0.25 M	1
8	7.0	sodium phosphate	0.29 M	0
9	7.5	sodium phosphate	0.29 M	0
10	7.5	tris	0.29 M	0

[0025] All formulations form good lyophilized cakes and reconstitute within 20-30 seconds.

[0026] Table IV summarizes recovery of activity and specific activity after several months and at the three storage temperatures. The data for the 4°C samples after three months is similar for most of the formulations except formulations 8 and 10 which lost activity. After three months, at 30°C, formulations 2, 8, 9, and 10 lost activity. The greatest recovery of activity and specific activity is seen for formulations 1, 3, 5, 6, and 7.

[0027] Table V summarizes increase in aggregation over time. At 4°C, after three months, formulations 1-7, have <4% increase in HMW, and at 30°C, formulations 8, 9, and 10 show highest aggregate formation. At 30°C, formulation 1 shows no increase in HMW, even after four months, with all the other formulations showing >3% HMW. Formulations 2, 8, 9, and 10 (all containing no sucrose) show elevated aggregate formation.

Table IV

Percent Recovery of Activity/Specific Activity at Three Different Storage Temperatures and Times

	TIME/TEMPERATURE															
	-80°C						4°C				30°C					
Sample Number	One Month		3 Months		4 Months		One Month		3 Months		One Month		3 months		4 Months	
	Act.	Spec. Act.	Act.	Spec. Act.	Act.	Spec. Act.	Act.	Spec. Act.	Act.	Spec. Act.	Act.	Spec. Act.	Act.	Spec. Act.	Act.	Spec. Act.
1	100	92	103	92	100	99	110	110	110	109	100	100	100	97	93	95
2	100	90	98	100	ND	ND	104	108	83	88	54	55	44	52	ND	ND
3	112	110	90	90	92	89	105	108	97	102	99	100	85	95	89	90
4	85	90	85	84	ND	ND	91	95	81	90	84	92	58	64	ND	ND
5	94	96	99	95	95	90	92	96	107	106	85	90	83	92	80	78
6	100	102	96	100	ND	ND	100	102	92	100	91	96	77	85	ND	ND
7	100	102	100	100	ND	ND	110	110	100	112	111	115	86	97	ND	ND
8	105	105	92	94	ND	ND	78	90	64	72	50	70	0	ND	ND	ND
9	110	110	94	98	ND	ND	100	102	84	94	48	60	26	41	ND	ND
10	90	90	84	80	ND	ND	70	85	21	26	0	0	0	ND	ND	ND

All percentages are expressed as a percentage of the Time Zero, which in this case is "Post-lyophilization" and is set equal to 100%. Recoveries greater than 100% reflect assay variability.
Spec. Act. = Specific Activity (U/mg)
Act. = Activity (U/ml) = Clotting Activity
ND = not determined

Table V

(%) HMW vs. Time Lyophilized							
		4°C			30°C		
Sample No.	Time Zero	1 Month	3 Months	4 Months	1 Month	3 Months	4 Months
1	0.6	0.1	0.0	0.1	0.1	0.2	0.3
2	0.6	0.3	0.5	ND	2.8	6.3	ND
3	0.5	0.5	1.4	1.8	1.0	3.5	4.0
4	0.6	0.6	1.9	ND	1.2	3.9	ND
5	0.5	0.0	0.3	1.2	0.4	3.0	5.1
6	0.6	0.7	2.6	ND	1.4	4.7	ND
7	0.9	0.9	3.3	ND	1.9	4.2	ND
8	1.6	9.4	12.1	ND	16.0	18.6	ND
9	1.8	5.9	8.3	ND	20.0	34.5	ND
10	0.8	3.3	21.5	ND	76.0	72.0	ND
ND = not determined							

Example 3

[0028] To minimize the volume requirements of shipping containers, it is preferable to concentrate the bulk protein as much as possible (*e.g.*, up to at least 20 mg/ml) prior to shipping to a fill/finish facility. Moreover, it is desirable to have the bulk drug product and finished product in similar formulations.

[0029] To evaluate concentrated preparations of factor IX, useful as bulk drug product, twelve formulations were prepared as indicated in Table VI below, except at high (≥ 10 mg/ml) factor IX concentrations. The surfactant concentration is either about 0.005 or 0.02% Tween-80® (useful as a Tween optimization study). All samples have factor IX at a concentration of ≥ 10 mg/ml and sucrose at 1%. The osmolality of all samples was 300 ± 50 mOsM.

Table VI

Factor IX Formulations - Bulk High Concentration				
Sample No.	pH	Buffer (10 mM)	Salts	% Tween-80
1A	7.0	histidine	0.26 M glycine	.005
1B	7.0	histidine	0.26 M glycine	.02
2A	7.0	sodium phosphate	0.25 M glycine	.005
2B	7.0	sodium phosphate	0.25 M glycine	.02
3A	7.0	potassium phosphate	0.25 M glycine	.005
3B	7.0	potassium phosphate	0.25 M glycine	.02
4A	7.5	tris	0.26 M glycine	.005
4B	7.5	tris	0.26 M glycine	.02
5A	7.5	potassium phosphate	0.25 M glycine	.005
5B	7.5	potassium phosphate	0.25 M glycine	.02
6A	7.5	sodium phosphate	0.25 M glycine	.005
6B	7.5	sodium phosphate	0.25 M glycine	.02

[0030] The samples are subjected to five freeze-thaw cycles, repeated freezing at - 80°C, subsequent thawing at 37°C for five cycles, and analyzed for recovery of total factor IX concentration, activity, and specific activity. The level of factor IX (mg/ml) ranges from 10.40 to 15.20 mg/ml. The initial percent HMW is <0.5%. There is no loss of protein

or activity, and no significant increase in aggregate formation from the freeze-thaw cycles for the 12 formulations. The high concentration formulated bulk product for several formulations of Table V are analyzed for stability after storage at -80°C for one month. No increase in % HMW is observed and the specific activity is maintained.

[0031] Numerous modifications and variations in the invention as described in the above illustrative examples are expected to occur to those skilled in the art and, consequently, only such limitations as appear in the appended claims should be placed thereon. Accordingly, it is intended in the appended claims to cover all such equivalent variations which come within the scope of the invention as claimed.

Claims

1. A composition being stable to lyophilization comprising factor IX, glycine, sucrose, and a surfactant, with the proviso that the composition is isotonic.
2. The composition of claim 1 , further comprising calcium chloride.
3. The composition of any one of claims 1 or 2, wherein said surfactant is polysorbate.
4. The composition of any one of claims 1 to 3 further comprising a buffering agent.
5. The composition of any one of claims 1 to 4, wherein said buffering agent is a member selected from the group consisting of histidine, phosphate, tris, and diethanolamine.
6. The composition of any one of claims 1 to 5, comprising factor IX, glycine, histidine, sucrose and polysorbate.
7. The composition of any one of claims 1 to 5, comprising factor IX, glycine, sodium phosphate, sucrose, and polysorbate.
8. The composition of any one of claims 1 to 5, comprising factor IX, glycine, potassium phosphate, sucrose, and polysorbate.
9. The composition of any one of claims 1 to 5, comprising factor IX, glycine. tris, sucrose, and polysorbate.
10. The composition of, any one of claims 1 to 5, comprising factor IX, glycine, calcium chloride, polysorbate, and sucrose.
11. The composition of any one of claims 1 to 5, comprising factor IX, glycine, tris, calcium chloride, polysorbate, and sucrose.
12. The composition of any one of claims 1 to 5, comprising factor IX, glycine, diethanolamine, polysorbate, and sucrose.
13. The composition of any one of claims 1 to 12, wherein said factor IX concentration is about 0.4 to 20 mg/ml.
14. The composition of claim 13, wherein said factor IX concentration is about 0.1 to 10 mg/ml.
15. The composition of any one of claims 1 to 14, wherein said factor IX concentration is about 0.5 to 10 mg/ml.
16. The composition of any one of claims 1 to 15, wherein said glycine concentration is about 0.1 to 0.3 M.
17. The composition of claim 16, wherein said glycine concentration is about 0.2 to 0.3 M.
18. The composition of claim 17, wherein said glycine concentration is about 0.26 M.
19. The composition of any one of claims 4 to 18, wherein said buffering agent is about 5 to 30 mM histidine.
20. The composition of claim 19, wherein said histidine concentration is about 10 mM.

21. The composition of any one of claims 1 to 20, wherein the sucrose concentration is about 0.5 to 2 %.

22. The composition of claim 21, wherein said sucrose concentration is about 1 %.

23. The composition of any one of claims 1 to 22, wherein said surfactant is about 0.005 to 0.05 % polysorbate.

24. The composition of claim 23, wherein said polysorbate concentration is about 0.005 %.

25. The composition of any one of claims 1 to 24, comprising 0.75 mg/ml factor IX, 0.26 M glycine, 10 mM histidine, 1 % sucrose, and 0.005 % polysorbate.

Patentansprüche

1. Zusammensetzung, die beim Lyophilisieren stabil ist und Faktor IX, Glycin, Saccharose und ein Tensid enthält, mit der Maßgabe, dass die Zusammensetzung isotonisch ist.

2. Zusammensetzung nach Anspruch 1, weiterhin umfassend Calciumchlorid.

3. Zusammensetzung nach einem der Ansprüche 1 oder 2, wobei das Tensid Polysorbat ist.

4. Zusammensetzung nach einem der Ansprüche 1 bis 3, weiterhin umfassend ein Puffermittel.

5. Zusammensetzung nach einem der Ansprüche 1 bis 4, wobei das Puffermittel ein Mitglied ist ausgewählt aus der Gruppe bestehend aus Histidin, Phosphat, Tris und Diethanolamin.

6. Zusammensetzung nach einem der Ansprüche 1 bis 5, die Faktor IX, Glycin, Histidin, Saccharose und Polysorbat umfasst.

7. Zusammensetzung nach einem der Ansprüche 1 bis 5, die Faktor IX, Glycin, Natriumphosphat, Saccharose und Polysorbat umfasst.

8. Zusammensetzung nach einem der Ansprüche 1 bis 5, die Faktor IX, Glycin, Kaliumphosphat, Saccharose und Polysorbat umfasst.

9. Zusammensetzung nach einem der Ansprüche 1 bis 5, die Faktor IX, Glycin, Tris, Saccharose und Polysorbat umfasst.

10. Zusammensetzung nach einem der Ansprüche 1 bis 5, die Faktor IX, Glycin, Calciumchlorid, Polysorbat und Saccharose umfasst.

11. Zusammensetzung nach einem der Ansprüche 1 bis 5, die Faktor IX, Glycin, Tris, Calciumchlorid, Polysorbat und Saccharose umfasst.

12. Zusammensetzung nach einem der Ansprüche 1 bis 5, die Faktor IX, Glycin, Diethanolamin, Polysorbat und Saccharose umfasst.

13. Zusammensetzung nach einem der Ansprüche 1 bis 12, wobei die Konzentration des Faktors IX etwa 0,4 bis 20 mg/ml beträgt.

14. Zusammensetzung nach Anspruch 13, wobei die Konzentration des Faktors IX etwa 0,1 bis 10 mg/ml beträgt.

15. Zusammensetzung nach einem der Ansprüche 1 bis 14, wobei die Konzentration des Faktors IX etwa 0,5 bis 10 mg/ml beträgt.

16. Zusammensetzung nach einem der Ansprüche 1 bis 15, wobei die Glycinkonzentration etwa 0,1 bis 0,3 M beträgt.

17. Zusammensetzung nach Anspruch 16, wobei die Glycinkonzentration etwa 0,2 bis 0,3 M beträgt.

18. Zusammensetzung nach Anspruch 17, wobei die Glycinkonzentration etwa 0,26 M beträgt.

19. Zusammensetzung nach einem der Ansprüche 4 bis 18, wobei das Puffermittel etwa 5 bis 30 mM Histidin ist.

20. Zusammensetzung nach Anspruch 19, wobei die Histidinkonzentration etwa 10 mM beträgt.

21. Zusammensetzung nach einem der Ansprüche 1 bis 20, wobei die Saccharosekonzentration etwa 0,5 bis 2% beträgt.

22. Zusammensetzung nach Anspruch 21, wobei die Saccharosekonzentration etwa 1% beträgt.

23. Zusammensetzung nach einem der Ansprüche 1 bis 22, wobei das Tensid etwa 0,005 bis 0,05% Polysorbat ist.

24. Zusammensetzung nach Anspruch 23, wobei die Polysorbatkonzentration etwa 0,005% beträgt.

25. Zusammensetzung nach einem der Ansprüche 1 bis 24, die 0,75 mg/ml Faktor IX, 0,26 M Glycin, 10 mM Histidin, 1% Saccharose und 0,005% Polysorbat umfasst.

Revendications

1. Composition stable à la lyophilisation, comprenant du facteur IX, de la glycine, du saccharose et un agent tensio-actif, sous réserve que la composition soit isotonique.

2. Composition suivant la revendication 1, comprenant en outre du chlorure de calcium.

3. Composition suivant l'une quelconque des revendications 1 ou 2, dans laquelle ledit agent tensio-actif est un polysorbate.

4. Composition suivant l'une quelconque des revendications 1 à 3, comprenant en outre un agent tampon.

5. Composition suivant l'une quelconque des revendications 1 à 4, dans laquelle ledit agent tampon est un membre choisi dans le groupe consistant en histidine, phosphate, tris et diethanolamine.

6. Composition suivant l'une quelconque des revendications 1 à 5, comprenant du facteur IX, de la glycine, de l'histidine, du saccharose et du polysorbate.

7. Composition suivant l'une quelconque des revendications 1 à 5, comprenant du facteur IX, de la glycine, du phosphate de sodium, du saccharose et du polysorbate.

8. Composition suivant l'une quelconque des revendications 1 à 5, comprenant du facteur IX, de la glycine, du phosphate de potassium, du saccharose et du polysorbate.

9. Composition suivant l'une quelconque des revendications 1 à 5, comprenant du facteur IX, de la glycine, du tris, du saccharose et du polysorbate.

10. Composition suivant l'une quelconque des revendications 1 à 5, comprenant du facteur IX, de la glycine, du chlorure de calcium, du polysorbate et du saccharose

11. Composition suivant l'une quelconque des revendications 1 à 5, comprenant du facteur IX, de la glycine, du tris, du chlorure de calcium, du polysorbate et du saccharose.

12. Composition suivant l'une quelconque des revendications 1 à 5, comprenant du facteur IX, de la glycine, de la diéthanolamine, du polysorbate et du saccharose.

13. Composition suivant l'une quelconque des revendications 1 à 12, dans laquelle ladite concentration en facteur IX est comprise dans l'intervalle d'environ 0,4 à 20 mg/ml.

14. Composition suivant la revendication 13, dans laquelle ladite concentration en facteur IX est comprise dans l'intervalle d'environ 0,1 à 10 mg/ml.

15. Composition suivant l'une quelconque des revendications 1 à 14, dans laquelle ladite concentration en facteur IX est comprise dans l'intervalle d'environ 0,5 à 10 mg/ml.

16. Composition suivant l'une quelconque des revendications 1 à 15, dans laquelle ladite concentration en glycine est comprise dans l'intervalle d'environ 0,1 à 0,3 M.

17. Composition suivant la revendication 16, dans laquelle ladite concentration en glycine est comprise dans l'intervalle d'environ 0,2 à 0,3 M.

18. Composition suivant la revendication 17, dans laquelle ladite concentration en glycine est égale à environ 0,26 M.

19. Composition suivant l'une quelconque des revendications 4 à 18, dans laquelle ledit agent tampon consiste en histidine à une concentration d'environ 5 à 30 mM.

20. Composition suivant la revendication 19, dans laquelle ladite concentration en histidine est égale à environ 10 mM.

21. Composition suivant l'une quelconque des revendications 1 à 20, dans laquelle la concentration en saccharose est comprise dans l'intervalle d'environ 0,5 à 2%.

22. Composition suivant la revendication 21, dans laquelle ladite concentration en saccharose est égale à environ 1%.

23. Composition suivant l'une quelconque des revendications 1 à 22, dans laquelle ledit agent tensio-actif consiste en polysorbate à une concentration d'environ 0,005 à 0,05%.

24. Composition suivant la revendication 23, dans laquelle ladite concentration en polysorbate est égale à environ 0,005%.

25. Composition suivant l'une quelconque des revendications 1 à 24, comprenant 0,75 mg/ml de facteur IX, 0,26 M de glycine, 10 mM d'histidine, 1% de saccharose et 0,005% de polysorbate.

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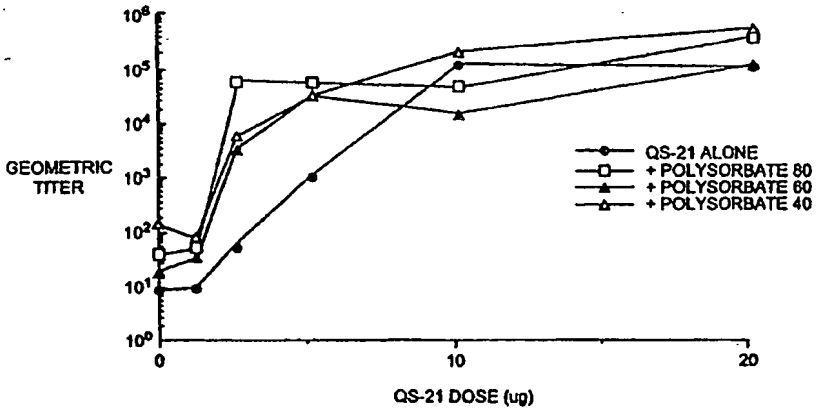

(57) Abstract

Certain novel compositions of the adjuvant saponin QS-21 having improved properties are disclosed. The compositions of the present invention are designed (1) to minimize the lytic effects of QS-21, (2) to improve the tolerance of QS-21 containing formulations in humans or other animals, (3) to stabilize the QS-21 from alkaline hydrolysis and/or (4) to maintain the high adjuvant potency of the QS-21 product. These compositions may be employed with vaccines comprising proteins or peptides, polysaccharides, lipids, or nucleic acids.

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COMPOSITIONS COMPRISING THE ADJUVANT QS-21 AND POLYSORBATE OR CYCLODEXTRIN AS EXIPIENT

FIELD OF THE INVENTION

The present invention relates to the field of immune adjuvants and the use thereof as immune adjuvants in vaccines. The compositions of the present invention exhibit significantly improved properties relevant to the lytic effect, tolerance to QS-21 associated pain, and product stability of QS-21, and maintain full adjuvant activity.

BACKGROUND OF THE INVENTION

Adjuvant saponins have been identified and purified from an aqueous extract of the bark of the South American tree, *Quillaja saponaria* Molina. Among the 22 peaks which were separable and displayed saponin activity, QS-21 was one of the more predominant purified saponins. This saponin has been substantially purified by high pressure liquid chromatography (HPLC), low pressure liquid silica chromatography, and hydrophilic interactive chromatography (HILIC). QS-21 has been found to be useful as an immune adjuvant for enhancing immune responses in individuals at a much lower concentration than the previously available heterogeneous saponin preparations without the toxic effects associated with crude saponin preparations.

QS-21 is a membrane-lytic triterpene glycoside saponin. It forms micelles of approximately the same radius as bovine serum albumin (Kensil, U.S. Patent No. 5,057,540) and has a critical micellar concentration of approximately 50 µg/ml in PBS (Soltysik, S., et al., 1995, Vaccine 13:1403-1410).

The potency of an adjuvant formulation containing an antigen plus QS-21 can be assessed in experiments that address the relationship of adjuvant dose to immunological function (dose-response experiments). A decrease in adjuvant potency is expected to increase the minimum dose (threshold dose) required for enhancement of immune response. A desirable composition is expected to maintain an equivalent or better potency than the formulation that is used as a reference. For QS-21, the reference formulation is a simple solution in phosphate-buffered saline (PBS) or saline.

The adjuvant activity of QS-21 is assessed in animal models such as mice. The primary responses measured are increases in antigen-specific antibody and antigen-specific cytotoxic T lymphocytes (CTL). The threshold dose of QS-21 that will enhance murine immune response (antibody or CTL) has been measured in simple buffer solution such as PBS. A dose of 2.5 μg has been shown to be the threshold dose for antibody (Kensil, C.R., et al., 1993, Vaccine Research 2:273-281) and for CTL (Newman, M.J., et al., 1992, J. Immunology 148:2357-2362) to the antigen ovalbumin (OVA) in C57BL/6 mice in PBS. Similar threshold doses were observed when aluminum hydroxide was included in the PBS formulation (Kensil, C.R., et al., 1993, Vaccine Research 2:273-281). However, it is expected that there may be differences in potency between different compositions of a given adjuvant.

Despite these beneficial qualities, QS-21 possesses some unwelcome qualities as well. For instance, QS-21 associates with phospholipid bilayers and causes a lytic effect on certain cell membranes (i.e., erythrocytes). QS-21 will absorb to the phospholipid bilayer of sheep erythrocytes and cause the red blood cells to release hemoglobin. This hemoglobin release, which is known as

hemolysis, occurs at a concentration of approximately 5-7 μg/ml in a simple buffer such as saline or PBS (Kensil, C.R., et al., 1991, J. Immunology 146:431-437). At higher concentrations (above the critical micellar concentration of QS-21), total lysis of the red blood cell membrane occurs. The lytic effect of QS-21 is, therefore, an undesirable property for a composition.

In *in vivo* studies, hemolysis is not noted. However, after intramuscular injection of QS-21/saline solutions into New Zealand white rabbits, mild to moderate fibroblast damage or necrosis is noted in some animals when the injection site is analyzed histopathologically (Kensil, C.R., et al., 1995, In: Vaccine Design: The Subunit and Adjuvant Approach, Powell, M.F. and Newman, M.J., Eds., Plenum Press, NY). Further, creatine kinase, a marker for muscle damage is increased after injection with QS-21 in saline or PBS. This rise is believed to be due to the lytic effect of QS-21 on cell membranes.

Moreover, in clinical trials, some individuals have experienced an immediate, transient pain after injection with QS-21 in simple buffer solutions (saline or PBS). This pain, described by most individuals as a burning pain, may be a secondary reaction correlated with the lytic effect of the QS-21 adjuvant. Patient pain is likewise an objectionable property for a composition.

Product stability is another concern for QS-21 containing compositions. The shelf life of a vaccine product is typically defined by the extent of time to reach a defined and acceptable low level of degradation (such as, the time to 10% degradation, also known as t_{90}). Most commercial vaccine products have a shelf life of at least 18 to 24 months when stored in the refrigerator at 4°C. Adjuvants, which are essential components of vaccines, therefore must also have equally long shelf lives. However, the shelf life of a 50 μg/ml solution of

QS-21 at pH 7.0 at 4°C is reached in about 3 months. The reason for the short shelf life is because the ester bond of QS-21 is increasingly labile at increasing pH and because monomers of QS-21, as opposed to micelles, are subject to hydrolysis. The need to stabilize compositions of QS-21 adjuvant is significant.

SUMMARY OF THE INVENTION

A need exists for compositions of the saponin adjuvant QS-21 that may be used to boost the antigenic immune response in a relatively low dose with low local reactions and side effects, but also features a reduced lytic effect, improved tolerance to QS-21, and an increased stability. Accordingly, the present invention provides novel compositions of QS-21 that have these improved characteristics compared to a simple solution of QS-21 in a buffer such as saline or PBS. Surprisingly, the full adjuvant potency of QS-21 in the disclosed compositions is not compromised compared to a control formulation of QS-21 in PBS.

DESCRIPTION OF THE FIGURES

Figure 1 depicts a graph showing the adjuvant potency of various compositions. Figure 1A shows the effect of Polysorbate 40, Polysorbate 60, and Polysorbate 80 on the immune response of Balb/c mice to ovalbumin at different concentrations of QS-21. Figure 1B shows the effect of methyl-β-cyclodextrin on the immune response of Balb/c mice to ovalbumin at different concentrations of QS-21.

Figure 2 depicts a graph showing the effect of Polysorbate 80 and hydroxypropyl-β-cyclodextrin on Type 14 IgG3 antibody response to a

T-independent polysaccharide antigen.

Figure 3 shows a bar graph of patients' tolerance to pain for various excipients in QS-21 adjuvant compositions from Trial 1. This figure shows how the pain scores are classified as no pain, mild pain, moderate pain, or severe pain, where 0=no pain, 1-3=mild pain, 4-7=moderate pain, and 8-10=severe pain.

Figure 4 shows the individual scores for the patients' tolerance to pain in Figure 3. This figure shows individual immediate pain scores after injection of a given formulation on a scale of 0-10, where 0 is no pain and 10 is maximum pain.

Figure 5 shows a bar graph of patients' tolerance to pain for various excipients in QS-21 adjuvant compositions from Trial 2. This figure shows how the pain scores are classified as no pain, mild pain, moderate pain, or severe pain, where 0=no pain, 1-3=mild pain, 4-7=moderate pain, and 8-10=severe pain.

Figure 6 shows the individual scores for the patients' tolerance to pain in Figure 5. This figure shows individual immediate pain scores after injection of a given formulation on a scale of 0-10, where 0 is no pain and 10 is maximum pain. Mean and median scores for each formulation are listed below each formulation.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The saponins of the present invention may be obtained from the tree *Quillaja saponaria* Molina.

The term "saponin" as used herein includes glycosidic triterpenoid

compounds which produce foam in aqueous solution, have hemolytic activity in most cases, and possess immune adjuvant activity. The invention encompasses the saponin per se, as well as biologically active fragments thereof.

The invention also concerns compositions, such as immunologic compositions, comprising one or more substantially pure saponin fractions, and methods of using these compositions as immune adjuvants.

More particularly, the compositions of the present invention may reduce the *in vitro* lytic effects of a saponin adjuvant containing formulation. Another preferred composition is one that may maintain the maximum adjuvant activity of a saponin. Yet another preferred composition may increase the stability of a saponin adjuvant containing composition from alkaline hydrolysis. Other compositions may preferably improve an individual's tolerance to saponin adjuvant associated pain from a formulation containing a saponin adjuvant.

As described in Kensil, et al., U.S. Patent No. 5,057,540, the contents of which are fully incorporated by reference herein, the adjuvant activity of such saponins may be determined by any of a number of methods known to those of ordinary skill in the art. The increase in antibody titer of antibody against specific antigen upon administration of an adjuvant may be used as a criteria for adjuvant activity. (Dalsgaard, *Acta Verterinia Scandinavica*, 69:1 (1978); Bomford, *Int. Archs. Allergy Appl. Immun.* 77:409 (1985).) Briefly, one such test involves injecting CD-1 mice intradermally with an antigen (for instance, i.e., bovine serum albumin, BSA) mixed with varying amounts of the potential adjuvant. Sera was harvested from the mice two weeks later and tested by ELISA for anti-BSA antibody.

"QS-21" designates the mixture of isomeric components QS-21-V1 and QS-21-V2 which appear as a single peak on reverse phase HPLC on Vydac C4 (5 µm particle size, 300Å pore, 4.6 mm ID x 25 cml) in 40 mM acetic acid in methanol/water (58/42, v/v). The component fractions are referred to specifically as QS-21-V1 and QS-21-V2 when describing experiments performed on the further purified components.

The term "substantially pure" means substantially free from compounds normally associated with the saponin in its natural state and exhibiting constant and reproducible chromatographic response, elution profiles, and biologic activity. The term "substantially pure" is not meant to exclude artificial or synthetic mixtures of the saponin with other compounds.

The substantially pure QS-7 saponin also referred to as QA-7 in U.S. Patent No. 5,057,540) is characterized as having immune adjuvant activity, containing about 35% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205-210 nm, a retention time of approximately 9-10 minutes on RP-HPLC on a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 52-53% methanol form a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 10 mM ID x 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of approximately 0.06% (w/v) in water and 0.07% (w/v) in phosphate buffered saline, causing no detectable hemolysis of sheep red blood cells at concentrations of 200 µg/ml or less, and containing the monosaccharide residues terminal rhamnose, terminal xylose, terminal glucose, terminal

galactose, 3-xylose, 3,4-rhamnose, 2, 3-fucose, and 2,3-glucuronic acid, and apiose (linkage not determined).

The substantially pure QS-17 saponin (also referred to as QA-17 in U.S. Patent N. 5, 057, 540) is characterized as having immune adjuvant activity, containing about 29% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205-210 nm, a retention time of approximately 35 minutes on RP-HPLC on a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 63-64% methanol from a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 10 mM ID x 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of approximately 0.06% (w/v) in water and 0.03% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at 25 µg/ml or greater, and containing the monosaccharide residues terminal rhamnose, terminal xylose, 2-fucose, is characterized as having immune adjuvant activity, containing about 35% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205-210 nm, a retention time of approximately 9-10 minutes on RP-HPLC on a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 52-53% methanol form a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 10 mM ID x 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of approximately 0.06% in water and 0.07% in phosphate buffered saline, causing no detectable

hemolysis of sheep red blood cells at concentrations of 200 µg/ml or less, and containing the monosaccharide residues terminal rhamnose, terminal xylose, 2-fucose, 3-xylose, 3,4-rhamnose, 2,3-glucuronic acid, terminal glucose, 2-arabinose, terminal galactose and apiose (linkage not determined).

The substantially pure QS-18 saponin (also referred to as QA-18 in U.S. Patent No. 5,057,540) is characterized as having immune adjuvant activity, containing about 25-26% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205-210 nm, a retention time of approximately 38 minutes on RP-HPLC on a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 64-65% methanol from a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 10 mM ID x 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of approximately 0.04% (w/v) in water and 0.02% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at 25 µg/ml or greater, and containing the monosaccharide residues terminal arabinose, terminal apiose, terminal xylose, terminal glucose, terminal galactose, 2-fucose, 3-xylose, 3,4-rhamnose, and 2,3-glucuronic acid.

The substantially pure QS-21 saponin (also referred to as QA-21 in U.S. Patent No. 5,057,540) is characterized as having immune adjuvant activity, containing about 22% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205-210 nm, a retention time of approximately 51 minutes on RP-HPLC on a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 4.6 mm ID x 25 cm L in a solvent of 40 mM acetic acid

in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 69-70% methanol from a Vydac C_4 column having a 5 µm particle size, 300 Å pore, 10 mM ID x 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of approximately 0.03% (w/v) in water and 0.02% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at 25 µg/ml or greater. The component fractions, substantially pure QS-21-V1 and QS-21-V2 saponins, have the same molecular weight and identical spectrums by FAB-MS. They differ only in that QS-21-V1 has a terminal apiose which is xylose in QS-21-V2 (which therefore has two terminal xyloses and no apiose). The two components additionally contain the monosaccharides terminal arabinose, terminal apiose, terminal xylose, 4-rhamnose, terminal galactose, 2-fucose, 3-xylose, and 2,3-glucuronic acid.

The invention may also encompass impure forms of saponin adjuvants. For example, one preferred embodiment is the heterogenic saponin adjuvant known as "Quil A." Commercial preparations of Quil A are available from Superfos (Vedbaek, Denmark) and have been isolated from the bark of the South American tree, *Quillaja saponaria* Molina. Quil A is characterized chemically as carbohydrate moieties in glycosidic linkage to the triterpenoid quillaic acid. Quil A possesses immune adjuvant activity and separates into 20 discrete peaks by RP-HPLC on Vydac C_4 column having a 5 µm particle size, 300 Å pore, 4.6 mM ID x 25 cm L in a solvent of 40 mM acetic acid in methanol water (U.S. Patent No. 5,057,540).

The invention also relates to a composition which comprises a saponin adjuvant of the present invention, an antigen, and an excipient. Preferably, the

adjuvant is QS-21. Preferably, the excipients may be nonionic surfactants, polyvinylpyrollidone, human serum albumin, aluminum hydroxide, agents with anesthetic action, and various unmodified and devivatized cyclodextrins. More preferably, the nonionic surfactants may include Polysorbate 20, Polysorbate 40, Polysorbate 60, and Polysorbate 80. The polyvinylpyrollidone may preferably be Plasdone C15, a pharmaceutical grade of polyvinylpyrollidone. The agent having anesthetic action preferably is benzyl alcohol. A preferred cyclodextrin is a hydroxypropyl-β-cyclodextrin, which reduces QS-21 lysis of red blood cells *in vitro*.

The term "immune adjuvant," as used herein, refers to compounds which, when administered to an individual or tested *in vitro*, increase the immune response to an antigen in the individual or test system to which said antigen is administered. Preferably, such individuals are humans, however, the invention is not intended to be so limiting. Any animal that may experience the beneficial effects of the vaccines of the invention are within the scope of animals which may be treated according to the claimed invention. Some antigens are weakly immunogenic when administered alone or are toxic to the individual at concentrations which evoke immune responses in said individual. An immune adjuvant may enhance the immune response of the individual to the antigen by making the antigen more strongly immunogenic. The adjuvant effect may also lower the dose of said antigen necessary to achieve an immune response in said individual.

The saponins of the present invention may be utilized to enhance the immune response to any antigen. Typical antigens suitable for the immune-response provoking compositions of the present invention include antigens

derived from any of the following: viruses, such as influenza, feline leukemia virus, feline immunodeficiency virus, HIV-1, HIV-2, rabies, measles, hepatitis B, or hoof and mouth disease, bacteria, such as anthrax, diphtheria, Lyme disease or tuberculosis; or protozoans, such as *Babeosis bovis* or Plasmodium. The antigens may be proteins, peptides, polysaccharides, lipids, or nucleic acids encoding the protein or peptide. The proteins, peptides, lipids, or nucleic acids may be purified from a natural source, synthesized by means of solid phase synthesis, or may be obtained means of recombinant genetics.

Administration of the compounds useful in the method of the present invention may be by parenteral, intravenous, intramuscular, subcutaneous, intranasal, oral or any other suitable means. The dosage administered may be dependent upon the age, weight, species, kind of concurrent treatment, if any, route of administration, and nature of the antigen administered. In general, the saponin and antigen may be administered at a dosage of about 0.001 to about 1.0 mg/kg of saponin adjuvant or antigen per weight of the individual. The initial dose may be followed up with a booster dosage after a period of about four weeks to enhance the immunogenic response. Further booster dosages may also be administered.

The effective compound useful in the method of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. The vaccine of the present invention may be administered parenterally, intranasally, or orally.

Another preferred embodiment is a method for reducing the *in vitro* lytic effect of an immune adjuvant composition comprising administering to an

individual an effective amount of QS-21 and an excipient. Preferably, the excipients may be nonionic surfactants, polyvinylpyrollidone, human serum albumin, aluminum hydroxide, agents with anesthetic action, and various unmodified and devivatized cyclodextrins. More preferably, the nonionic surfactants may include Polysorbate 20, Polysorbate 40, Polysorbate 60, and Polysorbate 80. The polyvinylpyrollidone may preferably be Plasdone C15, a pharmaceutical grade of polyvinylpyrollidone. The agent having anesthetic action preferably is benzyl alcohol. A preferred cyclodextrin is Encapsin, a hydroxypropyl-β-cyclodextrin, which reduces QS-21 lysis of red blood cells *in vitro*.

Other preferred methods falling within the scope of the invention include a method for maintaining the maximum adjuvant activity of QS-21 comprising administering to an individual an effective amount of QS-21 and an excipient and a method for improving the tolerance to saponin adjuvant associated pain in an individual to whom it is administered comprising administering an effective amount of QS-21 and an excipient.

EXAMPLES

A variety of excipients were evaluated in combination with QS-21 as novel compositions. These included various nonionic surfactants (Triton X-100, Polysorbate 20, Polysorbate 40, Polysorbate 60, and Polysorbate 80), polyvinylpyrollidone (Plasdone C15), human serum albumin, aluminum hydroxide, agents with anesthetic action (benzyl alcohol), and various unmodified and derivatized cyclodextrins (hydroxypropyl-β-cyclodextrin, hydroxypropyl-γ-cyclodextrin, methyl-β-cyclodextrin). The final formulations

were assessed for their capacity to reduce the lytic effect of QS-21, to improve tolerance to QS-21 adjuvant associated pain in humans, to stabilize QS-21 in aqueous solution, and/or to maintain maximum adjuvant potency relative to a control formulation of QS-21 in PBS.

Example 1

Compositions that Reduce the Lytic Effect of QS-21

A simple *in vitro* assay was used to screen excipients for reducing the lytic effect of QS-21. The lytic effect of QS-21 can be determined in an assay of hemolysis of sheep erythrocytes. Briefly, various two fold serial dilutions of QS-21 in a given excipient are prepared in a round bottom microtiter plate (100 µl/well). All plates contain control wells containing excipient, but no QS-21. The concentration of QS-21 ranges from 1.56 to 200 µg/ml. A total volume of 25 µl of sheep erythrocytes (washed with PBS) is added to each well, mixed with the QS-21/excipient solution, and incubated at ambient temperature for 30 minutes. After the end of the incubation, the round bottom plate is centrifuged at 2000 rpm for 5 minutes to sediment any unlysed cells. A total volume of 75 µl of supernatant (containing released hemoglobin) is transferred to the equivalent well of a flat-bottom 96 well plate. The flat-bottom plate is centrifuged at 2000 rpm for 5 minutes to break any air bubbles. The absorbance at 570 nm is read in a microtiter plate reader. Absorbance at 570 nm is plotted on the y-axis against QS-21 concentration plotted on the x-axis. The absorbance of hemoglobin in the supernatant of a well where no intact cell pellet was observed is defined as maximum hemolysis. The hemolytic index of QS-21 is defined as the concentration of QS-21 that yields

an absorbance equivalent to 50% of the maximum absorbance. An excipient that reduces the lytic effect of QS-21 is expected to increase the hemolytic index.

Table 1 lists the hemolytic indices of QS-21 in various excipients. All excipients were tested in the absence of QS-21. In the absence of QS-21, no hemolysis was noted, indicating that the excipient formulations were isotonic. Excipients that were shown to be effective in minimizing the lytic effect (increase hemolytic index) of QS-21 were hydroxypropyl-β-cyclodextrin, aluminum hydroxide, and Polysorbate 80 in saline.

Table 1:

Excipient	Hemolytic Index (µg/ml)
PBS	5
α-cyclodextrin (2 mg/ml)	1.5
β-cyclodextrin (2 mg/ml)	10
methyl-β-cyclodextrin (2 mg/ml)	36
hydroxypropyl-γ-cyclodextrin (2 mg/ml)	5
hydroxypropyl-β-cyclodextrin (1 mg/ml)	9
hydroxypropyl-β-cyclodextrin (2 mg/ml)	11
hydroxypropyl-β-cyclodextrin (4 mg/ml)	18
hydroxypropyl-β-cyclodextrin (8 mg/ml)	32
hydroxypropyl-β-cyclodextrin (16 mg/ml)	51
hydroxypropyl-β-cyclodextrin (32 mg/ml)	93
human serum albumin (40 mg/ml)	9
QS-7 (250 µg/ml)	30
aluminum hydroxide (2 mg/ml) in PBS	5
aluminum hydroxide (2 mg/ml) in saline	13
Monophosphoryl lipid A (25 µg/ml)	4.9
Monophosphoryl lipid A (50 µg/ml)	7.7
Monophosphoryl lipid A (100 µg/ml)	6.5
Triton X-100 (50 µg/ml)	1
Triton X-100 (100 µg/ml)	1
Polysorbate 80 (2 mg/ml)	9
Polysorbate 80 (4 mg/ml)	18
Polysorbate 80 (10 mg/ml)	38

Example 2

Compositions that Reduce the Lytic Effects of Other Saponins

Other saponin adjuvants are also known to be hemolytic, although to different extent than QS-21. These saponins include substantially pure QS-7, QS-17, and QS-18. In addition, heterogeneous adjuvant saponins such as Quil A are hemolytic. An example of the effect of Polysorbate 80 and hydroxypropyl-β-cyclodextrin on the hemolytic indices of the substantially pure QS-7 and heterogeneous Quil A is shown in Table 2. Hydroxypropyl-β-cyclodextrin was shown to be effective in reducing the lytic effect (increasing the hemolytic index) of QS-7. Polysorbate 80 and hydroxypropyl-β-cyclodextrin were shown to be effective in minimizing the lytic effect (increasing the hemolytic index) of Quil A.

Table 2:

Saponin	Excipient	Hemolytic Index (µg/ml)
QS-7	PBS	650
QS-7	Polysorbate 80 (8 mg/ml)	60
QS-7	Hydroxypropyl-β-cyclodextrin (32 mg/ml)	>1000
Quil A	PBS	18
Quil A	Polysorbate 80 (8 mg/ml)	43
Quil A	Hydroxypropyl-β-cyclodextrin (32 mg/ml)	200

Example 3

Compositions that Stabilize QS-21

QS-21 is an acylated bidesmodic triterpene saponin. It has a fatty acid ester linked to the hydroxyl residues of fucose. In aqueous solution, this fatty

acid ester migrates between two adjacent vicinal hydroxyl groups (fucose 3, 4) to form two equilibrium isomers (Jacobsen, N.E., Fairbrother, W.J., et al., 1996, Carbohydrate Research 280:1-14). The predominant isomer is acylated at fucose 4 and the minor isomer is acylated at fucose 3. This ester bond is the most labile bond in QS-21 and will hydrolize under alkaline conditions to form a deacylated saponin and a fatty acid-arabinose domain. The deacylated saponin and the fatty acid domain are both inactive as immunological adjuvants (Kensil, C.R., et al., 1996, In: Saponins Used in Traditional and Modern Medicine, Waller and Yamaski, Eds., Plenum Press, NY, 165-172). Various conditions affect the stability of this ester bond (Cleland, J.L., et al., 1996, J. Pharmaceutical Sciences 85:22-28). Furthermore, the monomer form of QS-21 is more susceptible to hydrolysis than the micellar form.

Examples of the shelf life of QS-21 are shown in Table 3. The aqueous shelf life for a 50 µg/ml QS-21 solution at pH 7.0 at 4°C was shown to be only 94 days or approximately 3 months. This is representative of a typical clinical vaccine formulation containing QS-21 adjuvant (which consists of QS-21 at a concentration of 50-200 µg/ml in a physiological pH buffer (pH 7.0-7.5)). Hence, in simple buffer and salt solutions at low concentration, the QS-21 product does not maintain a desirable stability profile. Some improvement in stability, however, can be achieved by an increased concentration of the QS-21 product. For instance, the shelf life of a 500 µg/ml QS-21 solution at pH 7.0 at 4°C was shown to be 717 days, or 23.9 months. But a concentrated QS-21 solution is not necessarily a practical method of administering a low dose of adjuvant. For example, administration of 25 µg from a 500 µg/ml solution would require the syringe withdrawal of 0.05 ml of dose. Additionally, some

improved stability can be achieved by the use of a lower pH, i.e., at pH 6.0. However, a pH substantially lower than the physiological pH range may not be tolerated well or be compatible with the antigen.

Table 3:

QS-21 Concentration	pH	t_{90} (days)
50 µg/ml	pH 7.0	94
50 µg/ml	pH 6.0	679
500 µg/ml	pH 7.0	717

Another way to evaluate the stability of QS-21 in aqueous solution was to assay the solution by HPLC in an accelerated stability assay at 37°C. Although this is not the temperature used for storage of vaccines (4°C), it was expected that this assay at 37°C would show the relative stabilizing power of a given excipient. For example, an excipient that extended the t_{90} value by two fold at 37°C would also be expected to extend the t_{90} value by two fold at 4°C.

Specifically, QS-21 (100 µg/ml) was prepared in various excipients in PBS at pH 7.0. The solutions were incubated at 37°C for 7 days. At the end of 7 days, the solutions were assayed by reversed phase-HPLC to determine the extent of degradation. The data was plotted as log (fraction QS-21 t=7/QS-21 t=0 days) against time on the x-axis. The time to 10% degradation (t_{90}) was extrapolated from this plot.

Table 4 shows the t_{90} values of QS-21 in various excipients. Stabilization of QS-21 is shown by an increase in t_{90}. Excipients that stabilized QS-21 by at least two fold are Polysorbate 20, Polysorbate 80, native *Quillaja* saponin QS-7, and the deacylsaponin resulting from alkaline hydrolysis of QS-21 (DS-1).

Table 4:

Excipient	t_{90} (days) at 37°C
PBS (pH 7.0)	1.2
Polysorbate 20 (720 µg/ml)	2.9
Polysorbate 80 (250 µg/ml)	3.2
Polysorbate 80 (500 µg/ml)	4.3
Polysorbate 80 (1.0 mg/ml)	5.2
Polysorbate 80 (2.0 mg/ml)	7.2
Phenol (2.5 mg/ml)	2.3
Pluronic F68 (1.0 mg/ml)	1.4
QS-7 (100 µg/ml)	1.8
QS-7 (250 µg/ml)	2.6
QS-7 (500 µg/ml)	9.0
QS-7 (1.0 mg/ml)	16.0
DS-1 (100 µg/ml)	2.2
DS-1 (250 µg/ml)	3.3
DS-1 (500 µg/ml)	7.2
DS-1 (1.0 mg/ml)	6.2
Monocaproyl-rac-glycerol (1.0 mg/ml)	1.7
α-cyclodextrin (5 mg/ml)	0.8
β-cyclodextrin (5 mg/ml)	0.7
Methyl-β-cyclodextrin (5 mg/ml)	1.5
hydroxypropyl-γ-cyclodextrin (5 mg/ml)	1.0
hydroxypropyl-β-cyclodextrin (5 mg/ml)	1.0

In addition, 0.9% benzyl alcohol, and Plasdone C15 were evaluated for its ability to stabilize QS-21 (Table 5). All QS-21 concentrations and incubation conditions were equivalent in this experiment except that the QS-21 formulation was prepared in Dulbecco's PBS (without calcium or magnesium)

at pH 7.5. As expected, the higher pH resulted in a faster degradation of QS-21 in PBS. However, Plasdone C15 stabilized QS-21.

Table 5:

Excipient	t_{90} (days) at 37°C, pH 7.5
Dulbecco's PBS	0.6
0.9% benzyl alcohol in Dulbecco's PBS	0.7
Plasdone C15 in Dulbecco's PBS (25 mg/ml)	1.6
Plasdone C15 in Dulbecco's PBS (50 mg/ml)	7.7

Example 4

Adjuvant Potency of Compositions

Figures 1A and 1B show the effect of Polysorbate 40, Polysorbate 60, Polysorbate 80, and methyl-β-cyclodextrin on the immune response of Balb/c mice to OVA plus various doses of QS-21. Female mice (10/group, 8-10 weeks of age at the first immunization) were immunized subcutaneously with 5 μg of OVA and the indicated dose of QS-21 in either PBS alone or in 2 mg/ml excipient in PBS. A booster immunization was given by the same route at week 2. Sera was collected at week 4 for EIA analysis of the anti-OVA response. Mice were analyzed for OVA-specific IgG2a by a standard EIA analysis (Kensil, C.R., et al., 1993, Vaccine Research 2:273-281). QS-21 was active in all excipients within two fold of the threshold value determined in PBS. The same maximum level of antibody response was reached at the optimum adjuvant dose (typically 10 μg and above).

Figure 2 shows the effect of excipients on antibody response to a T-independent polysaccharide antigen. Balb/c mice were immunized subcutaneously with a commercial 23-valent *S. pneumonia* polysaccharide vaccine (Pnu-Imune, 0.5 μg/serotype) and different doses of QS-21 in PBS, in 4 mg/ml Polysorbate 80 in PBS, or in 16 mg/ml hydroxypropyl-β-cyclodextrin in PBS. Anti-Type 14 IgG was determined by EIA on sera collected at day 7 after a single immunization. Neither Polysorbate 80 or hydroxypropyl-β-cyclodextrin in the formulation reduced the potency of the vaccine for stimulating an IgG3 response specific for Type 14 polysaccharide serotype.

Example 5

Clinical Studies of Compositions-Trial 1

Various QS-21 compositions were administered to patients in order to test for the compositions' pain tolerance. Fifteen volunteers were recruited to receive four intramuscular injections, with each injection given at one week intervals. The study was carried out as a randomized, double-blind study. Three of the formulations contained 50 μg QS-21 in either Dulbecco's PBS (without calcium or magnesium), in 4 mg/ml Polysorbate 80 in PBS, or in 1 mg/ml aluminum hydroxide in saline. The fourth formulation was a PBS control without QS-21. Volunteers were asked to rate the immediate pain in the first five minutes after injection on a 0 to 10 scale (0=no pain, 1-3=mild, 4-7=moderate, 8-10=severe). The results are shown in Figure 3. The cumulative scores represented in Figure 3 of the patients' tolerance to pain is represented in Figure 4 as individual scores. The QS-21 formulation containing 4 mg/ml Polysorbate 80 resulted in an improved pain tolerance compared to QS-21 in

PBS. The highest score for this particular formulation was rated as a 5.

Example 6

Clinical Studies of Compositions-Trial 2

Various other QS-21 compositions were administered to patients in order to test for the compositions' pain tolerance. Fifteen volunteers were recruited to receive four intramuscular injections, with each injection given at one week intervals. The study was carried out as a randomized, double-blind study. The excipients evaluated were benzyl alcohol, hydroxypropyl-beta-cyclodextrin, and a higher dose of Polysorbate 80, which had been shown to be more effective than 4 mg/ml Polysorbate 80 at reducing QS-21 lysis of red blood cells *in vitro*. The five formulations tested were (1) 1 mg/ml aluminum hydroxide, which served as the placebo control; (2) 50 µg QS-21 in 0.72% benzyl alcohol in saline; (3) 50 µg QS-21 in 30 mg/ml hydroxypropyl-β-cyclodextrin; (Encapsin, Janssen Biotech N.V., Olen, Belgium) (4) 50 µg QS-21 in 8 mg/ml Polysorbate 80; and (5) 50 µg QS-21 in PBS (Dulbecco's PBS without calcium or magnesium), which served as a positive control formulation. Volunteers were asked to rate the immediate pain in the first five minutes after injection on a 0 to 10 scale (0=no pain, 1-3=mild, 4-7=moderate, 8-10=severe). The results are shown in Figure 5. The cumulative scores represented in Figure 5 of the patients' tolerance to pain is represented in Figure 6 as individual scores. All excipients were shown to reduce the mean and median pain scores associated with QS-21 in PBS. The highest single score for the QS-21/Encapsin formulation was rated as a 5, which compared more

favorably with the QS-21/Polysorbate 80 formulation that was rated with a single 6 and two 5's.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.

We claim:

1. A composition comprising an antigen, a saponin adjuvant, and an excipient, wherein the composition reduces the *in vitro* lytic effect of the saponin adjuvant.

2. The composition according to claim 1, wherein the saponin adjuvant is a substantially pure saponin adjuvant.

3. The composition according to claim 2, wherein the substantially pure saponin adjuvant is QS-7 or QS-21.

4. The composition according to claim 1, wherein the saponin adjuvant is a heterogenous saponin adjuvant.

5. The composition according to claim 4, wherein the heterogeneous saponin adjuvant is Quil A.

6. The composition according to claim 1, wherein the antigen is a peptide, a protein, a polysaccharide, a lipid, or a nucleic acid encoding the peptide or protein.

7. The composition according to claim 1, wherein the excipient is a nonionic surfactant.

8. The composition according to claim 7, wherein the nonionic surfactant is a Polysorbate.

9. The composition according to claim 8, wherein the Polysorbate is Polysorbate 20, Polysorbate 40, Polysorbate 60, or Polysorbate 80.

10. The composition according to claim 1, wherein the excipient is a cyclodextrin.

11. The composition according to claim 10, wherein the cyclodextrin is

β-cyclodextrin.

12. The composition according to claim 11, wherein the β-cyclodextrin is hydroxypropyl-β-cyclodextrin.

13. The composition according to claim 1, wherein the composition further maintains the maximum adjuvant activity of QS-21.

14. The composition according to claim 13, wherein the saponin adjuvant is a substantially pure saponin adjuvant.

15. The composition according to claim 14, wherein the substantially pure saponin adjuvant is QS-7 or QS-21.

16. The composition according to claim 13, wherein the saponin adjuvant is a heterogenous saponin adjuvant.

17. The composition according to claim 16, wherein the heterogeneous saponin adjuvant is Quil A.

18. The composition according to claim 13, wherein the antigen is a peptide, a protein, a polysaccharide, a lipid, or a nucleic acid encoding the peptide or protein.

19. The composition according to claim 13, wherein the excipient is a nonionic surfactant.

20. The composition according to claim 19, wherein the nonionic surfactant is a Polysorbate.

21. The composition according to claim 20, wherein the Polysorbate is Polysorbate 20, Polysorbate 40, Polysorbate 60, or Polysorbate 80.

22. The composition according to claim 13, wherein the excipient is a cyclodextrin.

23. The composition according to claim 22, wherein the cyclodextrin is β-cyclodextrin.

24. The composition according to claim 23, wherein the cyclodextrin is hydroxypropyl-β-cyclodextrin.

25. The composition according to claim 1, wherein the composition further has an increased stability.

26. The composition according to claim 25, wherein the saponin adjuvant is a substantially pure saponin adjuvant.

27. The composition according to claim 26, wherein the substantially pure saponin adjuvant is QS-7 or QS-21.

28. The composition according to claim 25, wherein the saponin adjuvant is a heterogenous saponin adjuvant.

29. The composition according to claim 28, wherein the heterogeneous saponin adjuvant is Quil A.

30. The composition according to claim 25, wherein the antigen is a peptide, a protein, a polysaccharide, a lipid, or a nucleic acid encoding the peptide or protein.

31. The composition according to claim 25, wherein the excipient is a nonionic surfactant.

32. The composition according to claim 31, wherein the nonionic surfactant is a Polysorbate.

33. The composition according to claim 26, wherein the Polysorbate is Polysorbate 20, Polysorbate 40, Polysorbate 60, or Polysorbate 80.

34. The composition according to claim 1, wherein the composition

further improves the tolerance to saponin adjuvant associated pain in an individual to whom it is administered.

35. The composition according to claim 34, wherein the saponin adjuvant is a substantially pure saponin.

36. The composition according to claim 35, wherein the substantially pure saponin adjuvant is QS-7 or QS-21.

37. The composition according to claim 34, wherein the saponin adjuvant is a heterogenous saponin adjuvant.

38. The composition according to claim 37, wherein the heterogeneous saponin adjuvant is Quil A.

39. The composition according to claim 34, wherein the antigen is a peptide, a protein, a polysaccharide, a lipid or a nucleic acid encoding the peptide or protein.

40. The composition according to claim 34, wherein the excipient is a nonionic surfactant.

41. The composition according to claim 40, wherein the nonionic surfactant is a Polysorbate.

42. The composition according to claim 41, wherein the Polysorbate is Polysorbate 20, Polysorbate 40, Polysorbate 60, or Polysorbate 80.

43. The composition according to claim 34, wherein the excipient is a cyclodextrin.

44. The composition according to claim 43, wherein the cyclodextrin is β-cyclodextrin.

45. The composition according to claim 44, wherein the cyclodextrin is

hydroxypropyl-β-cyclodextrin.

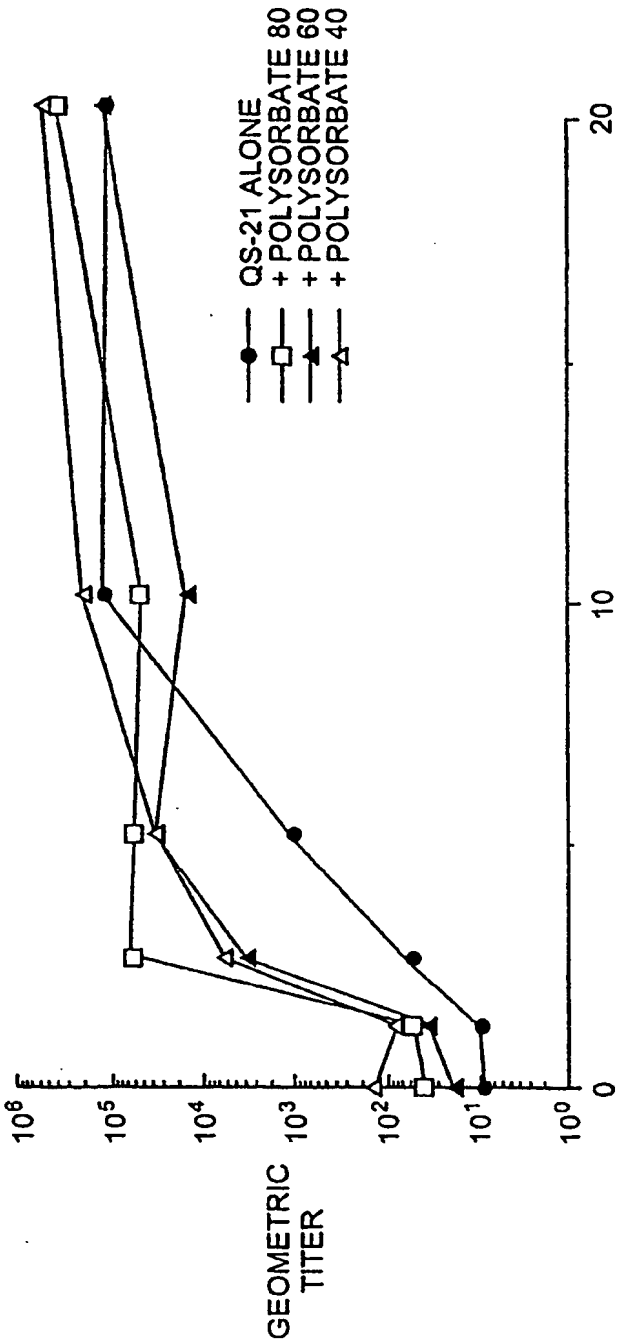
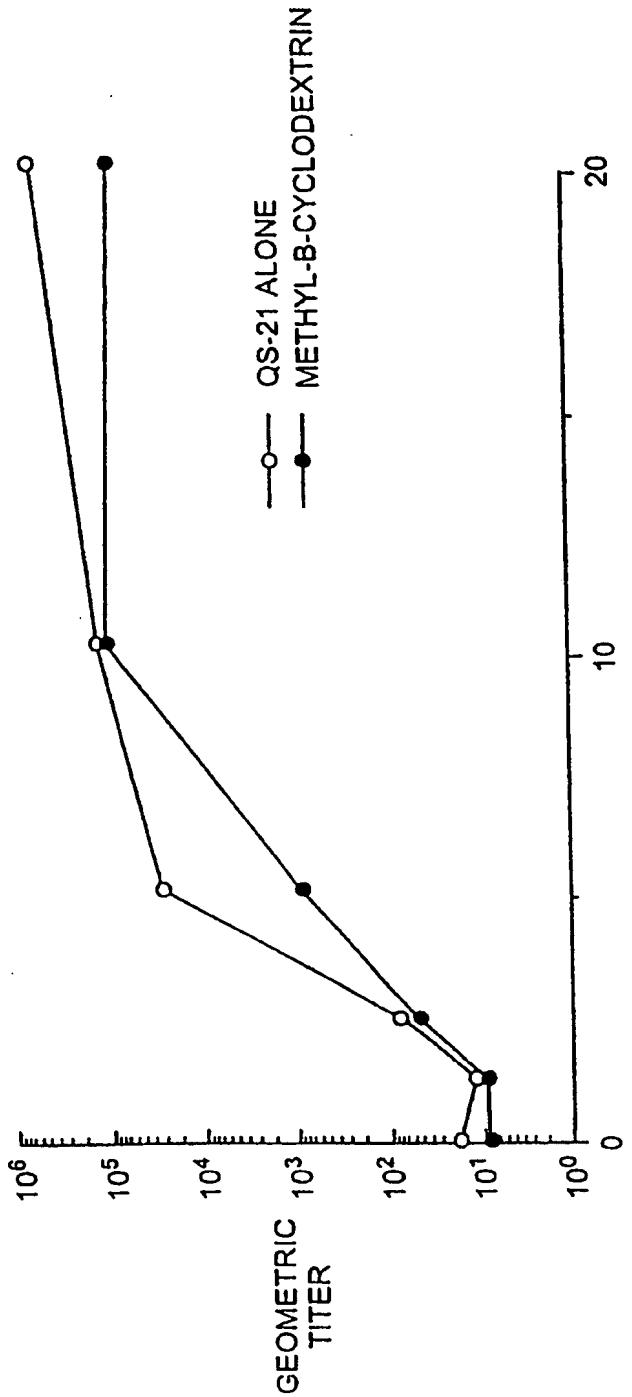

QS-21 ALONE
+ POLYSORBATE 80
+ POLYSORBATE 60
+ POLYSORBATE 40
GEOMETRIC TITER
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
10
20
QS-21 DOSE (ug)

FIG. 1A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METHYL-B-CYCLODEXTRIN
GEOMETRIC TITER
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10^5
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10^3
10^2
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10^0
0
10
20
QS-21 DOSE (ug)

FIG. 1B

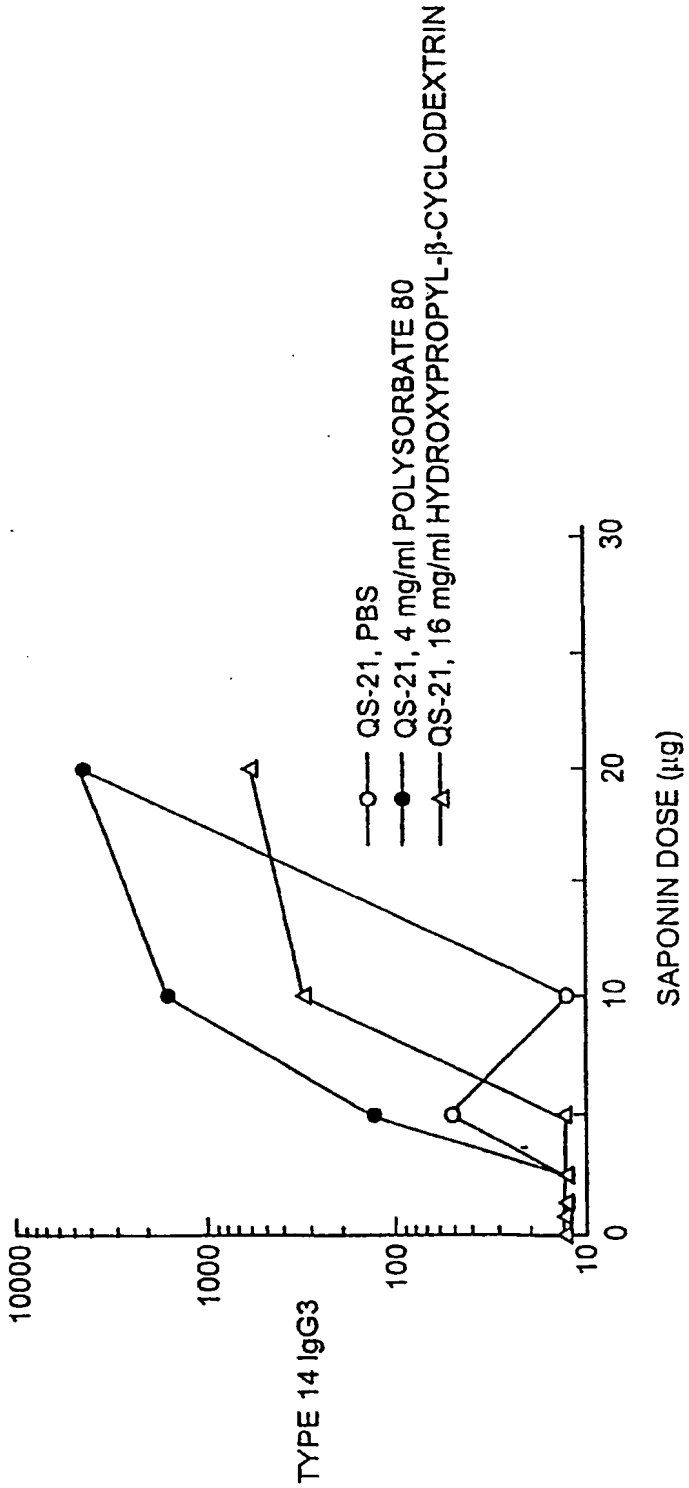

FIG. 2

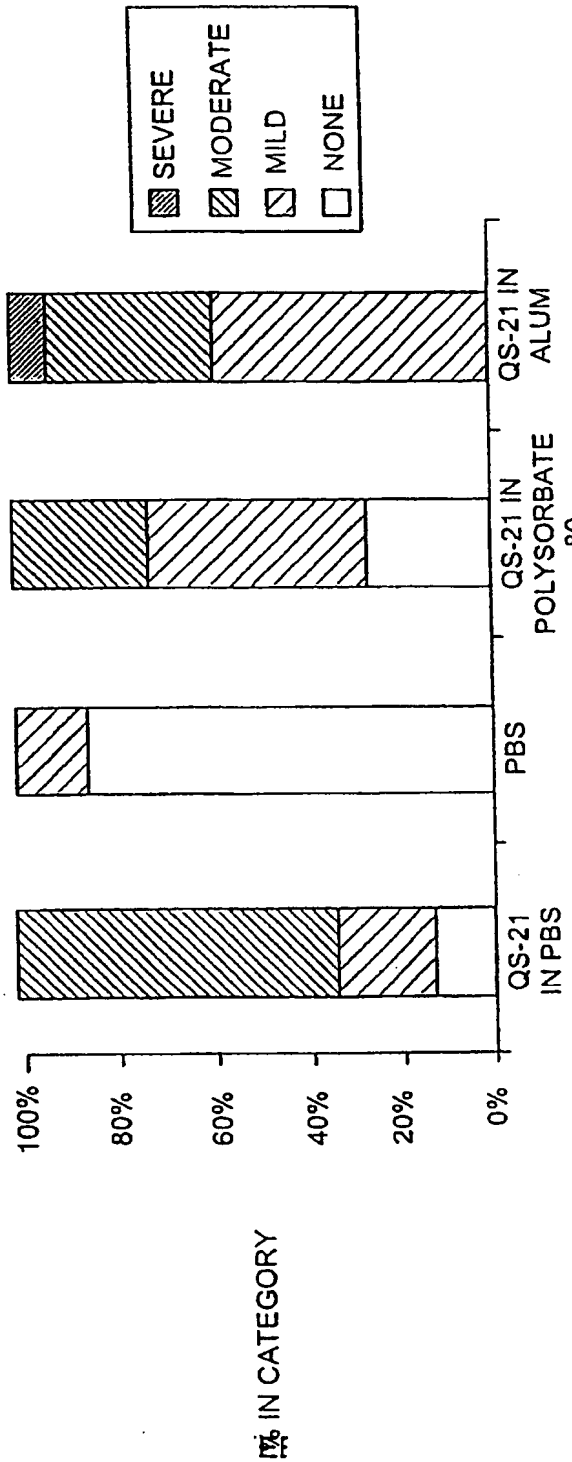
SEVERE
MODERATE
MILD
NONE
QS-21 IN ALUM
QS-21 IN POLYSORBATE 80
PBS
QS-21 IN PBS
100%
80%
60%
40%
20%
0%
% IN CATEGORY

FIG. 3

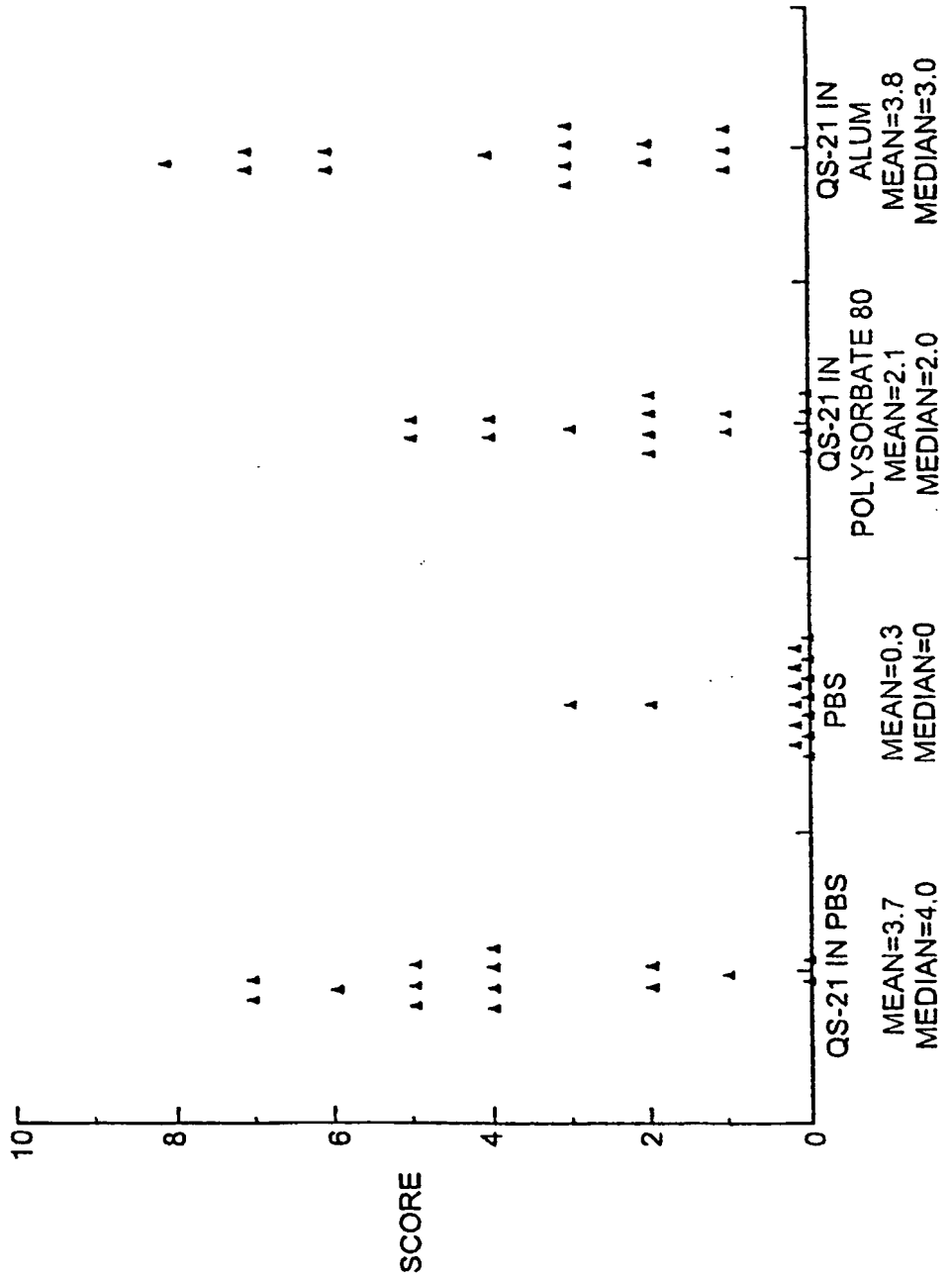

FIG. 4

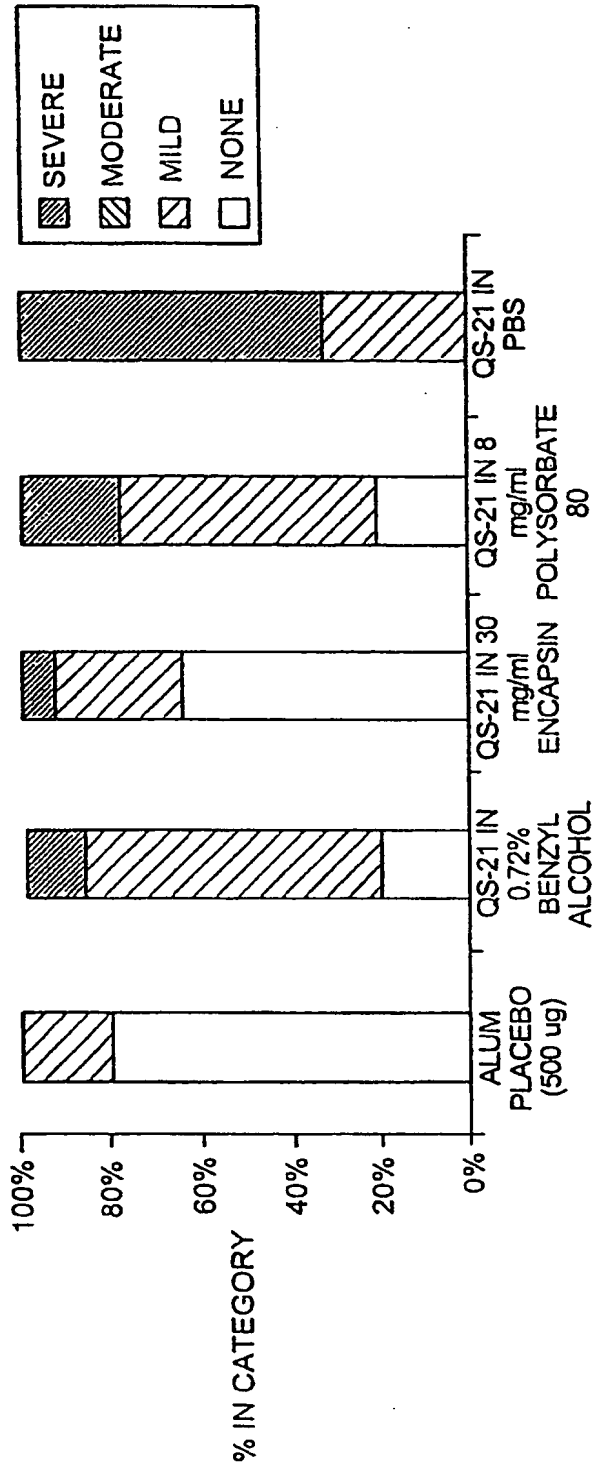
SEVERE
MODERATE
MILD
NONE
QS-21 IN PBS
QS-21 IN 8 mg/ml POLYSORBATE 80
QS-21 IN 30 mg/ml ENCAPSIN
QS-21 IN 0.72% BENZYL ALCOHOL
ALUM PLACEBO (500 ug)
100%
80%
60%
40%
20%
0%
% IN CATEGORY

FIG. 5

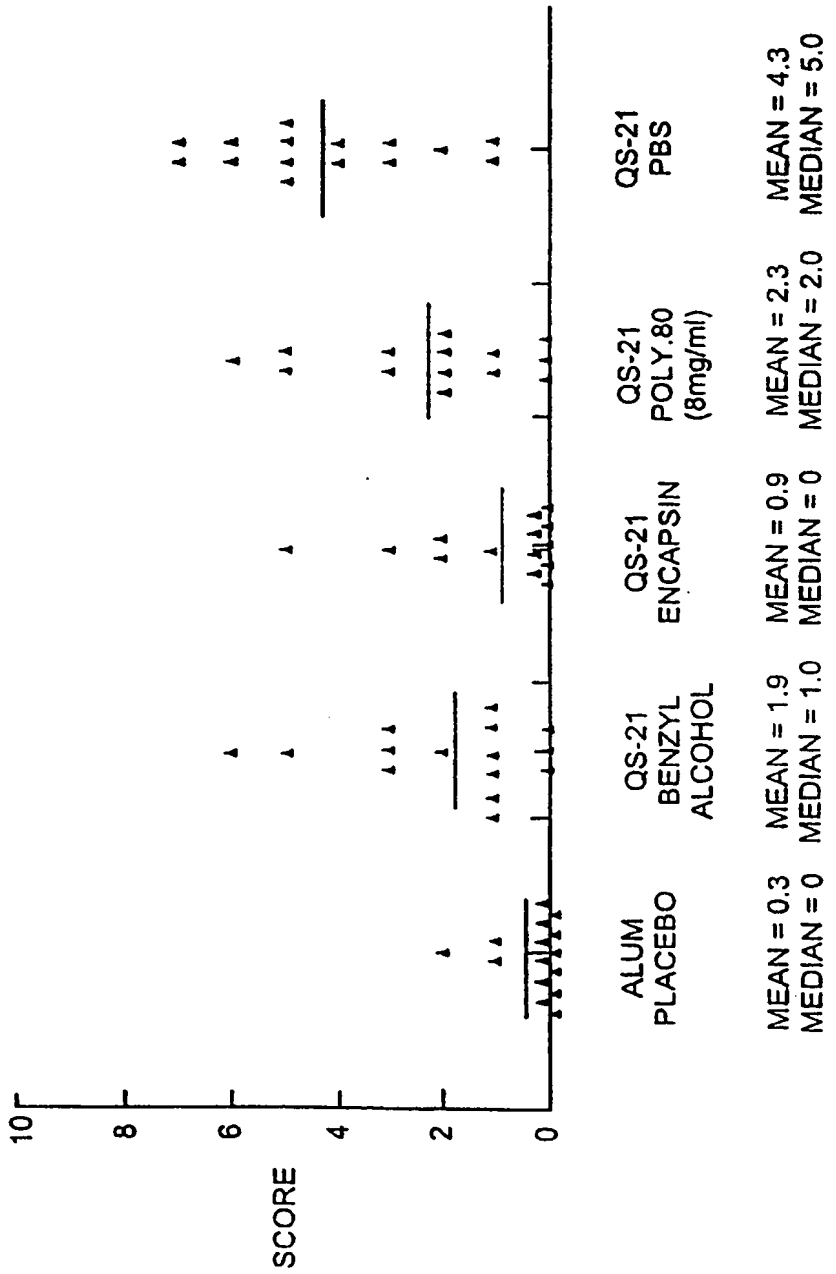

FIG. 6

INTERNATIONAL SEARCH REPORT

Int. onal Application No
PCT/US 98/17940

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 //(A61K39/39,47:40,47:34)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 650 398 A (SOLTYSIK SEAN ET AL) 22 July 1997 see column 1, line 12-17 see column 2, line 36-48 see column 6, line 10-24 see column 21, line 14-32 see column 22, line 3-36 see column 24, line 19-42 ---	1-9, 13-21, 25-42
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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 1999	01/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

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INTERNATIONAL SEARCH REPORT

Int. onal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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